MILK PROTEINS†

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Dr. Thomas Leroy McMeekin, a native of South Carolina, graduated from Clemson College with a B.S. in chemistry, and successively received his M.S. degree from Tulane, and his Ph.D. in biochemistry from the University of Chicago.

Dr. McMeekin was an assistant professor of biochemistry at Emory University for the years 1925-27. He then entered industrial work for a time, being engaged in the production of insulin for E. R. Squibb and Son in 1927-28. The next 12 years, 1928-1940, Dr. McMeekin was a research associate in the Department of Physical Chemistry, Harvard Medical School. In 1940, he became senior chemist in the Protein Division of the Eastern Regional Research Laboratory, in charge of the Production and Purification Section. Since 1947 he has been Head of the Protein Division.

THE IMPORTANCE of milk as a food and its availability have for a long time made milk proteins favorite proteins for investigation. In 1838, the great Dutch chemist, Mulder[11], who gave proteins their name, devised the method of separating casein by adding acid. It was considered to be a pure protein for almost a hundred years. Early work by Sebelien[19] demonstrated the complexity of the proteins of milk whey, obtained after the removal of casein. A globulin fraction was obtained by saturating whey with magnesium sulfate, and lactalbumin was prepared from the supernatant after the removal of globulin by acidification. Wichmann[24] reported the crystallization of lactalbumin from salt solutions. Although others have reported the crystallization of lactalbumin, it is now generally conceded that the crystalline B-lactoglobulin prepared by Palmer[14] constitutes the principal protein of the lactalbumin fraction. Osborne and his associates[13] devised methods for separating individual milk proteins, and with Wells[13] determined their purity by immunological means. Sorensen and Sorensen's[20] investigations on the albumin fraction of milk whey indicated that a large number of proteins with unique properties are present in small amounts.

Numerous empirical methods have been devised for separating and classifying milk proteins. Determination of nitrogen distribution of milk on the basis of the amount of protein separated by isoelectric precipitation, salt fractionation, and heat coagulation, has given comparative information. These studies showed that cow's milk contains about 3 percent casein and 0.7 percent other proteins.

Because of the biological significance of milk as a food, it was to be expected that milk proteins would have unique characteristics that would differentiate them from other tissue proteins. As compared with other proteins, casein is remarkably stable. In solution it may be heated, or treated with organic solvents, specific denaturing agents such as urea and guanidine hydrochloride, and small amounts of acid or alkali without apparent change in properties. *In vitro*, however, casein is digested with the greatest ease by proteolytic enzymes. It is well known that the ease of digestion of some proteins is greatly increased by denaturation or cooking[8], which appears to make a more accessible molecular structure by unfolding. Since casein cannot be denatured, it is frequently considered to be already denatured or to have an unfolded structure. Studies of physical properties of casein solutions such as viscosity and streaming birefringence are consistent with the idea that casein is a long molecule resembling denatured proteins.

To determine whether laboratory casein is in fact changed or denatured by the action of acid or alkali during its preparation, casein has been separated from milk at 2° by centrifugation at high velocities, according to the method of Ramsdell and Whittier[18], who isolated casein in its natural state from milk for the first time. The separated casein was converted into sodium caseinate by dialysis against a solution of sodium chloride, thus avoiding any change in acidity during its preparation. Measurements of optical rotation were used for detecting and measuring denaturation of casein, as it is well known that the denaturation of a protein is accompanied by a large increase in its negative specific rotation. A value of $[a]_D^{25}$ of −101 was obtained for the specific rotation of casein solutions, at pH 6.9 prepared without acid or alkali; this value is identical with that given by casein solutions prepared by means of acid and alkali. The rotations of both these caseins were unchanged by heating in solution. A slight increase in the value for the specific rotation of each was obtained when they were treated with 5-molar guanidine hydrochloride. When the guanidine hydrochloride was removed, however, the values for specific rotation returned to their previous values, showing that the effect of guanidine hydrochloride was that of the solution rather than a denaturation of the casein. In contrast, B-lactoglobulin, occurring in milk to the extent of only 0.4 percent behaves as a typical tissue protein in that it denatures easily; its specific rotation is increased from a value of $[a]_D^{25}$ of −43 at pH 8.5 to a value of −80 by heat, and to a value of −114 by guani-

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dine hydrochloride. These results suggest that casein occurs in milk in an unfolded configuration, which may be rapidly digested by proteolytic enzymes.

In undertaking a program of separating and purifying individual proteins of milk, it was important to determine whether the same proteins are always present in milk in the same relative amounts. The electrophoresis method of Tiselius was used for this study. The electrophoretic compositions of the proteins were determined on individual samples of milk obtained at regular intervals from four cows over their complete lactating cycles, including gestation. As shown in figure 1, there are four principal electrophoretic components in the proteins of skim milk. When casein was separated from whey by adjusting the pH to 4.7 and centrifuging, three of the electrophoretic components were found to be casein, amounting to about 80 percent of the total protein of milk. Since the proteins of whey amount to only 20 percent of the total protein of milk, it was desirable to separate them from casein before making electrophoretic determinations. The electrophoretic components of casein obtained from the milk of three cows were remarkably constant in area and mobilities throughout the lactating cycle. The electrophoretic components of some of the samples of casein from the fourth cow, however, varied, in that the *a*-casein component was split into two components. This is of interest in connection with the finding of Nitschmann and Lehmann[12] that the clotting of casein by rennet is associated with a split in the electrophoretic pattern of *a*-casein. Our results on the clotting of the separated components of casein showed that all the components of casein clot with rennet, and therefor a theory of clotting of casein based on the action of rennet on only one component is inadequate.

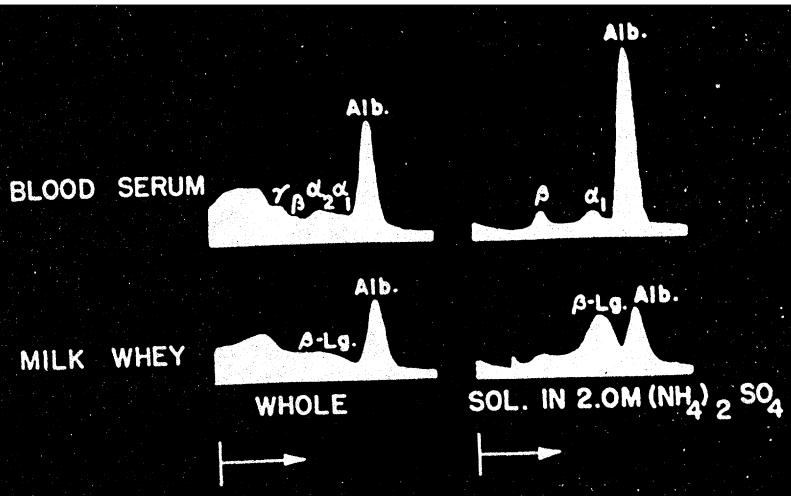

Fig. 2. Comparison of electrophoretic patterns of milk whey during the dry period (27 days before parturition) with the electrophoretic pattern of blood serum; veronal buffer, pH 8.35; ionic strength, 0.1. Alb., albumin; B-lg., B-lactoglobulin. Blood serum mobilities: -3.1 x10-5 (B-globulin); -5.2 x 10-5 (a, globulin); -6.8 x 10-5 (albumin). Milk whey mobilities: -2.8 x 10-5 (unidentified); -5.2 x 10-5 (B-lactoglobulin); -6.7 x 10-5 (albumin).

The relative amounts of the protein componets of whey varied considerably more than did the components of casein during the lactating cycle. The amount of colostrum globulin or immune globulin in whey increased markedly, beginning at 70 days before parturition. Also, the fast-moving component of whey, with the mobility of serum albumin, increased markedly at the end of the lactating cycle. This albumin component of milk has been crystallized by Polis, Shmukler, and Custer[16] from commercial mixed milk and shown to have the properties of blood serum albumin. Coulson and Stevens[2] demonstrated that this milk albumin is immunologically equivalent to blood serum albumin. As shown by the electrophoretic pattern, the whey fraction obtained from the cow's udders during the dry stage (about 27 days before parturition) contained a large amount of albumin. There is a marked similarity between the electrophoretic patterns of the whey proteins at this stage and those of the blood serum proteins (figure 2). The pH of the contents of the cow's udder at this stage is 7.4, the same as that of blood. On the day of the birth of the calf, however, the pH contents of the udder drop rapidly to the normal value of 6.6, presumably owing to the secretion of casein.

As shown by its electrophoretic pattern in figure 1, casein is a mixture of at least three components, which have been designated *a*-, B- and *r-casein* in the order of their decreasing mobilities. The chemical separation of these components has been a difficult task. All the

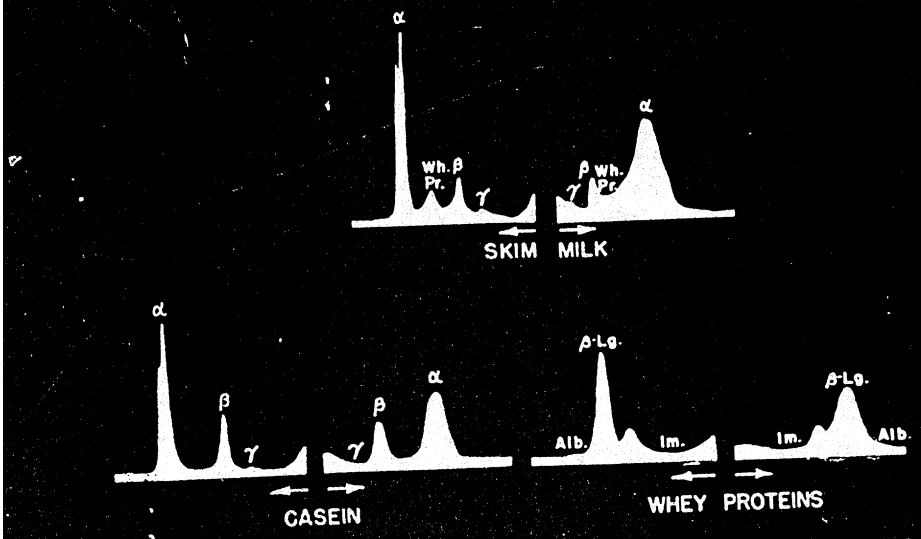

Fig. 1. Electrophoretic patterns of milk proteins in veronal buffer at a pH of 8.35 with an ionic strength of 0.1. Wh. pr., whey protein; B-Lg., B-lactoglobulin; Im., Immune; Alb., Albumin.

evidence, based on reproduction of properties of the unfractionated casein by mixing the separated components, shows that casein is a mixture. The properties of the mixture, however, are different from the properties of the pure components and give comfort to those who have felt that casein is a homogenous substance. Interaction between the components of casein is so pronounced that the properties of the individual components, such as solubility, are greatly modified when present in mixtures.

Three methods for separating the components of casein have been devised in our laboratory, and probably the success of each method is to a large extent due to finding conditions that minimize interaction between the components. The first of these methods was developed by Warner[23], who discovered that *a*- and B-casein could be separated by repeated reprecipitation from dilute solutions near the isoelectric point at 2°. Effective separations were obtained by this method. The method, however, is tedious, making the separation of the components in quantity difficult. The method by Hipp *et al.*[5], based on solubility in 50 percent alcohol in dilute salt solutions at different pH values, has been successfully used in separating the components of casein in larger amounts. Recently it has been found that the casein components can be most easily separated by means of urea solution. The properties and compositions of the components of casein prepared by these three methods are the same, indicating that casein is a mixture of proteins and that these components are not decomposition products.

Although the three caseins have the same general properties, such as insolubility at the isoelectric point, and are closely associated, they differ markedly in amino acid composition, as shown by Gordon *et al.*[3] in a complete amino acid analysis of these proteins. Calculations based on the amino acid content of these caseins reveal their relative polarity. *a*-casein contains 291 ionic groups and 965 nonpolar CH_2 groups per 10^5 grams, whereas *b*-casein contains 219 ionic groups and 1567 nonpolar CH_2 groups per 10^5 grams. If the ratio of ionic groups to the nonpolar CH_2 groups may be considered as a measure of polarity, then these calculations based on the amino acid composition indicate that *a*-casein is about twice as polar as B-casein. This calculation does not take into consideration the nonionic polar groups, because there are about an equal number in *a*- and B-casein. Polarity calculations, based on the ratio of the solubility in 50 percent alcohol to the solubility in water, also indicate that *a*-casein is about twice as polar as B-casein (6), thus demonstrating a relation between the amino acid composition and the solubility of these caseins.

After casein, B-lactoglobulin is the next most abundant protein in milk, constituting about 12 percent of the total protein. Milk also contains numerous other proteins in small quantities, some of which are enzymes. The isolation of B-lactoglobulin in crystalline form by Palmer[14] has furnished protein chemists with one of the most attractive proteins for investigation, in spite of the fact that it is electrophoretically inhomogeneous[7]. Although one of these components has been isolated in our laboratory[17], no difference in composition has been detected between the electrophoretically pure component and unfractionated B-lactoglobulin. Remarkably large B-lactoglobulin crystals can be obtained with ease by crystallizing from dilute salt solutions. McMeekin and Warner[9] determined directly the composition of B-lactoglobulin crystals suspended in water and concentrated ammonium sulfate solutions. The crystals were analyzed for water content by removing an individual crystal from the supernatant liquid. Surface liquid was removed by blotting, and the loss of water was determined by weight as a function of time. The water content of the crystal was obtained by subtracting the weight of the completely dried crystal from the weight of the crystal at the time of removal from the suspending liquid. The B-lactoglobulin crystals contained approximately 50 percent water rather than 20 percent, as given by the indirect method of Sorensen and Hoyrup[21]. Figure 3 illustrates the rate of loss of water by crystalline B-lactoglobulin when exposed to the air. It was found that the rate of loss of water follows a first-order equation, being proportional to the logarithm of the water remaining in the crystal until about 70 percent of the water is lost. This indicates that the vapor pressure of the water in the crystal does not change until most of the water is lost.

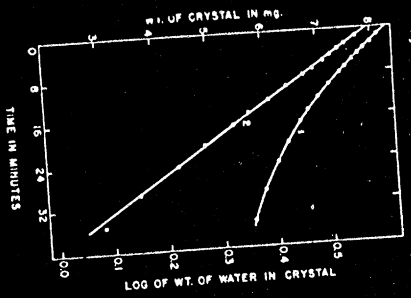

Fig. 3. Rate of loss of water by B-lactoglobulin crystal at room temperature. 1, loss in weight of crystal as a function of time; 2, logarithm of weight of water in the crystal shown in 1.

It was found also that ammonium sulfate went into B-lactoglobulin crystals, and on the basis of the water content of the crystal reached approximately 80 percent of the concentration of the suspending ammonium sulfate solution. In view of the results of Adair and Adair[1] based on density determinations, this finding was expected. The fact that salt diffuses into protein crystals accounts for the low results obtained for the water content of protein crystals by the method of Sorensen and Hoyrup.

To investigate the penetration of molecules into protein crystals, it is necessary to select conditions in which the protein crystal is relatively insoluble. B-lactoglobulin crystals are relatively insoluble in water and concentrations of ammonium sulfate greater than 2 molar. In more dilute solutions of ammonium sulfate, however, it is soluble. Consequently, sucrose, a nonelectrolyte in which B-lactoglobulin is relatively insoluble, was used to study the effect of concentration of the suspending medium on the composition of the protein crystal. The penetration of the protein crystal by sucrose was determined by density determinations on the crystal and by direct analysis. Figures 4 and 5 show the results. The density of the protein crystals was de-

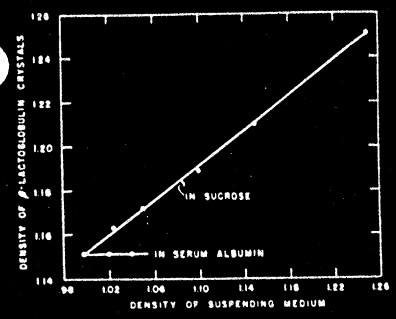

Fig. 4. Densities of B-lactoglobulin crystals equilibrated in sucrose hand serum albumin solutions.

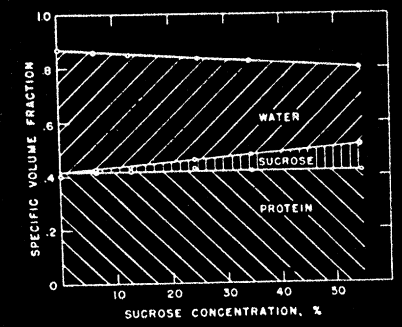

Fig. 5. Volume fractions of sucrose, protein, and water in crystals of B-lactoglobulin expressed as a function of concentration of sucrose in the suspending medium.

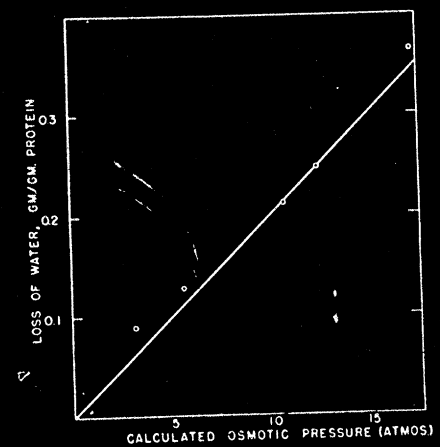

Fig. 6. Relationship between loss of water by B-lactoglobulin crystals and the osmotic pressure of the difference between sucrose in the crystal water and that in the suspending medium.

termined in bromobenzene-zylene mixtures after equilibrating in the suspending medium. Figure 4 illustrates that the density of the protein crystal is proportional to the density of the sucrose suspending medium. When the suspending medium was a solution of serum albumin, however, there was no increase in the density of the protein crystals. These results indicate that sucrose penetrates B-lactoglobulin crystals and that serum albumin, presumably because of its much larger size, does not.

The composition of the protein crystal is graphically illustrated in figure 5 as a function of sucrose concentration in the suspending medium. It may be seen that there is a reciprocal relationship between the amount of sucrose and water in the protein crystal The total volume of crystal, however, decreases with increasing concentrations of sucrose owing to loss of water not replaced by sucrose. That the forces controlling the distribution of water and sucrose in the protein crystal are largely osmotic is indicated by the straight line relation obtained when the loss in water by the crystal is plotted against osmotic pressure of the difference between the sucrose in the crystal water and that in the suspending medium (figure 6).

There are exceptions to these results which indicate that osmotic forces govern the composition of protein crystals suspended in solutions of small molecules. Thus when B-lactoglobulin crystals are suspended in saturated solutions of lithium bromide or chloride, the concentration of salt is greater in the crystal when calculated on the basis of crystal water than in the suspending medium. This result indicates that these lithium salts combine with the protein in the crystal. Sorensen and Palmer[22] have reported that the ammonium chloride content of B-lactoglobulin crystals suspended in dilute ammonium chloride is greater than in the suspending solution.

The water content of protein crystals has been considered to be of two kinds — bound and free[15]. The apparent "nonsolvent" water calculated from the difference in concentration of a reference substance in the water of the protein crystal and the suspending medium has been considered to be a measure of the bound water or hydration of the protein. Perutz[15] reported a value of 0.3 gram of water per gram of protein for the hydration of hemoglobin in ammonium sulfate solutions. The results of calculations of "nonsolvent" water in B-lactoglobulin crystals suspended in different concentrations of sucrose are shown in figure 7.

It is apparent from these results, based on large variations in sucrose concentration, that "nonsolvent" water values vary widely with concentration of sucrose and that there is no obvious basis for dividing the water of crystallization into two kinds. It appears plausible to relate the degree of hydration of protein crystals to the osmotic environment, in a manner similar to the relation of vapor-phase water absorption of proteins to vapor pressure. Thus, weakly polar groups such as the peptide bond may hold water loosely as suggested by Mellon *et al.*[10]. According to this view the hydration of a protein crystal varies with the osmotic environment. Previous studies on the "nonsolvent" water of protein crystals suspended in salt solutions have used such small changes in salt concentrations and vapor pressures that changes in hydration were not detected.

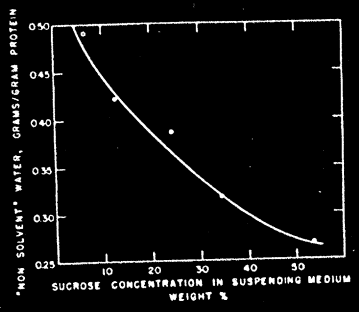

Fig. 7. Effect of sucrose concentration in the suspending medium on the "nonsolvent" water in B-lactoglobulin crystals.

When B-lactoglobulin crystals are equilibrated with water or a dilute salt solution, the solution tends to become more alkaline with time and show some indication of decomposition. This fact and other considerations led to a systematic study of the stability of B-lactoglobulin solutions as a function of pH and temperature. Groves *et al*[4] found that B-lactoglobulin denatures in relatively mild alkaline solutions and that the rate of denaturation increases rapidly with increase in alkalinity. Denaturation was followed by insolubility at the isoelectric point and by increase in optical rotation. When the loga-

rithm of the undenatured protein plotted against time, a straight line is obtained, as shown in figure 8, indicating that denaturation is a unimolecular reaction. The rate of denaturation was proportional to the first power of the hydrogen ion concentration between pH 8-10, as may be calculated from figure 9. This results suggests that pH denaturation involves one group in the B-lactoglobulin molecule. The surprising result was obtained that the rate of denaturation of B-lactoglobulin is the same at 3° as at 25°. The rate of denaturation of proteins, like other chemical reactions, usually increases rapidly with temperature. Solutions of crystalline B-lactoglobulin containing 2 molecules of the detergent dodecyl sulfate combined with each molecule of B-lactoglobulin are much more stable to alkali than without the detergent (figure 9). The rate of denaturation of this protein derivative has a normal temperature coefficient, being much greater at 25° than at 3°.

These results on the origin, separation, composition and properties of the proteins of milk constitute a brief summary of some of our studies on protein structure, uses and behavior.

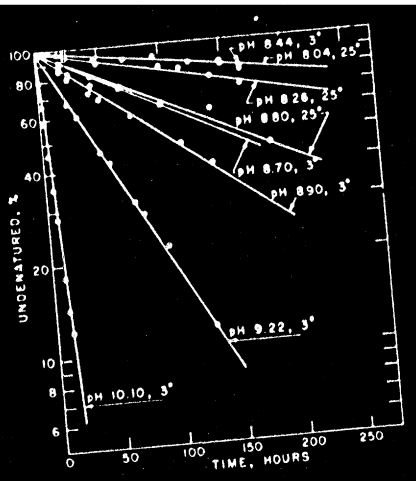

Fig. 8. Effect of pH on the velocity of denaturation of B-lactoglobulin in veronal buffer at 3 and 25°.

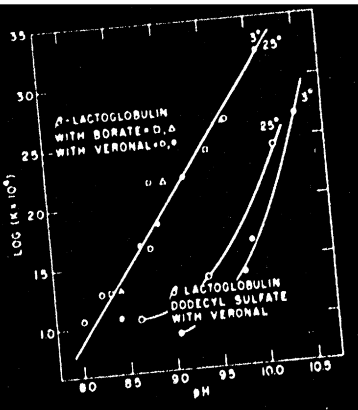

Fig. 9. Comparison of the effects of pH and temperature on the velocity constant of denaturation of B-lactoglobulin and B-lactoglobulin dodecyl sulfate. □, borate at 25°; △, borate at 3°; o, veronal at 25°; ●, veronal at 3°.

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MICHIGAN ASSOCIATION OF SANITARIANS

The Annual Meeting of the Michigan Association of Sanitarians will be held in the evening of April 9, 1952 at Michigan State College, East Lansing. One of the guest speakers will be Mr. H. L. Thomasson, President of the International Association of Milk and Food Sanitarians, who will talk on the advantages of belonging to the Association.

The meeting will be held at the same time as Ninth Annual Dairy and Food Sanitarians School of Michigan State College. The school will be held April 8, 9, 10, and 11. The program is as follows:

Sanitation Needs in the Dairy Industry ..... K. G. Weckel
Kinds of Microorganisms in Milk and their Behavior .. E. D. Devereux
Sources of Disease and Means of Transmission in Milk and Dairy Products ..... H. J. Stafseth
Plant Construction and Arrangement of Equipment ..... Horace Mitten
Odors and Tastes in Milk ..... G. M. Trout
The Manufacture of Dry Milk and Reconstituted Milk ..... J. Robert Brunner
Interpretation of Laboratory Findings ..... Clyde Smith
Balancing Inspection Services in the Dairy Industry .. H. L. Thomasson
The Manufacture of Ice Cream and Frozen Desserts .. Henry Kowalk
Contact with the Public:
The Interview .... Raymond Hatch
The Lecture ..... David Potter
Visual Aids — How to Prepare — How to Use — Evaluations of Various Types .... Wilbur Nelson
The School Lunch .... Mary Bodwell
Ventilation ..... Berney Blomfield
Personnel in the Food Establishment ..... H. S. Adams
Cooking of Foods and Food Handling ..... Pauline Paul
Bacteriology of Foods Frank Peabody
Refrigeration ..... W. L. Mallmann
Food Equipment — Demonstrations
A Self-planned and Enforced Sanitation Program at M.S.C.
The Viewpoint of Management ..... Emery G. Foster
The Program in Operation ..... Kenneth Lawson
The Meat Processing Industry ..... L. J. Bratzler
Balancing Inspection Services in the Food Industry ....W. L. Mallmann